15036

Relation Between Thiamine and Co-Carboxylase Concentration and Work Output of Perfused Frog Muscles.*

N. W. Shock and W. H. Sebrell.

From the Division of Physiology, National Institute of Health, U. S. Public Health Service, Bethesda, Md., and the Baltimore City Hospitals, Baltimore, Md.

The addition of 0.01 mM of thiamine hydrochloride per liter of perfusion fluid has been shown to increase significantly the work output of perfused frog muscles.[1] Observations on the relationship between thiamine concentrations in the perfusing fluid and the increase in work output are presented in the following report. In addition, the effectiveness of pyrophosphate thiamine or co-carboxylase in augmenting work output in perfused frog muscles has been assessed.

Experimental. The experimental methods used have been described in detail in a previous report.[1] Summarized briefly, fatigue curves were recorded for each gastrocnemius muscle of the frog under comparable conditions of perfusion, stimulation, loading, and recording. In the present experiments, stimulation was effected by single shocks from a Bird Electronic Inductorium operating through a voltage regulating transformer. Thiamine hydrochloride or co-carboxylase was added to the buffered glucose-Ringer's solution used for perfusion of the frog while the fatigue curve was recorded on the second gastrocnemius. Ten animals were tested with each concentration of thiamine or co-carboxylase. Thiamine concentration of the final perfusion fluid was varied from 0.0001 mM per liter to 1.5 mM per liter by adding appropriate amounts of a stock standard solution of thiamine containing 100 mM per liter. Co-carboxylase concentrations of 0.00001 to 0.1 mM per liter were prepared from a stock standard solution containing 50 mM per liter.† Individual work curves were plotted for each experiment and the total work done was estimated from the areas under the curves as determined with a planimeter.

Results. Effect of increasing concentration of thiamine on work output. The effect of adding 0.01 mM thiamine hydrochloride to the perfusing fluid is illustrated in Fig. 1. Fatigue curve A was first recorded from the left gastrocnemius muscle of a frog perfused with a buffered glucose Ringer's solution. The fatigue curve B was obtained on the right leg of the same animal after 15 minutes perfusion with the same buffered glucose Ringer's solution in which the thiamine concentration was .01 mM per liter. A marked improvement in work output of the muscle receiving thiamine is apparent. Total work output was calculated for each experiment as described previously,[1] and averages computed. The average difference in work output of the control and experimental legs obtained in 10 animals was calculated with its standard error.‡ Table I shows the average total work done by control and experimental legs with increasing concentration of thiamine. This table shows

* With the technical assistance of Frances Berg. A preliminary report was presented to Section N of the American Association for the Advancement of Science at the meeting held in Cleveland, September 10-11, 1944.

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† Thiamine hydrochloride and thiamine pyrophosphate, Merck, were used in preparing the stock solution by weight.

‡ The mean difference (experimental-control) was calculated for each group of 10 animals. The standard error of the mean difference was calculated from the distribution of differences as

$$\sigma_{\text{Mn. Diff.}} = \frac{\sqrt{\frac{\Sigma(\text{Exp.-Cont.})^2}{N} - (\text{Mn. Diff.})^2}}{\sqrt{N-1}}$$

and the critical ratio as

$$\text{C. R.} = \frac{\text{Mn. Diff.}}{\sigma_{\text{Mn. Diff.}}}$$

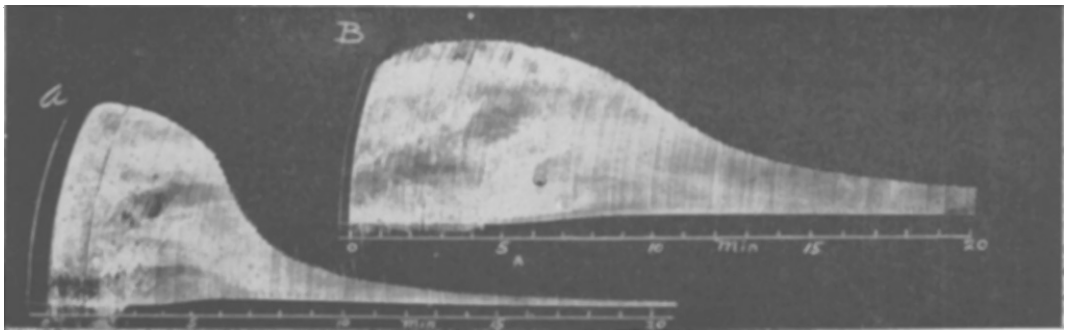

FIG. 1.

Improvement in work output of gastrocnemius muscle of perfused frog following the addition of 0.01 mM thiamine hydrochloride to perfusion fluid. Fatigue curve A of right gastrocnemius muscle during perfusion with buffered glucose-Ringer's solution. Fatigue curve B recorded from left leg of same frog after 15-minute perfusion with buffered glucose Ringer's solution containing 0.01 mM thiamine hydrochloride per liter. Time in minutes.

TABLE I.

Effect of Thiamine Concentration on Total Work Output of Perfused Frog Muscles.

Conc. of thiamine HCl mM/L		Avg work done (n = 10 animals)			C.R.†
		Cont. leg g cm	Exp. leg g cm	Mean difference* (Exp.-Cont.) g cm	
0.00	(a)‡	1449	1596	147 ± 93.0	1.6
0.00	(b)	2271	2421	150 ± 112.1	1.3
0.00	(c)	2302	2454	152 ± 73.0	2.0
0.0001		1793	1899	106 ± 69.7	1.5
0.0002		1884	2025	141 ± 80.5	1.8
0.0003		2177	2351	174 ± 122.8	1.4
0.0005		2281	2522	241 ± 87.5	2.8
0.0007		1824	2138	314 ± 103.6	3.0
0.00075		2315	2759	444 ± 36.0	12.3
0.001	(d)	2010	2428	418 ± 83.0	5.0
0.001	(e)	1553	1889	336 ± 95.1	3.5
0.005		1281	1538	257 ± 73.1	3.5
0.01		2042	2443	401 ± 97.3	4.1
0.1		2490	2823	333 ± 136.7	2.4
0.25		1958	2259	274 ± 86.6	3.2
0.5		2511	2868	357 ± 64.6	5.5
0.75		2533	2819	286 ± 129.4	2.2
1.0		2241	2460	219 ± 32.7	6.7
1.5		2196	2388	192 ± 57.5	3.3

* ±1 Standard error of the mean.

† Critical ratios greater than 3.0 are considered statistically significant. With 10 pairs of observations this criterion is equivalent to a P of about 0.01, which means that in only 1 out of 100 trials would one expect a difference as large or larger than the observed one to occur by chance (based on t table for small numbers).

‡ Groups a, b, c, d, and e as indicated in Discussion.

that the increase in work output observed at thiamine concentrations less than 0.0005 mM per liter were not statistically significant. Thiamine concentrations greater than 0.0005 mM per liter resulted in a significant improvement in work output of the treated muscle.

In Fig. 2 the average percentage increase in work output of the treated muscle over its untreated control is plotted against the logarithm of the concentration of thiamine in the perfusing fluid. This figure shows that the work output increases when the concentration of thiamine is increased from 0 to 0.001 mM per liter. With thiamine concentrations of

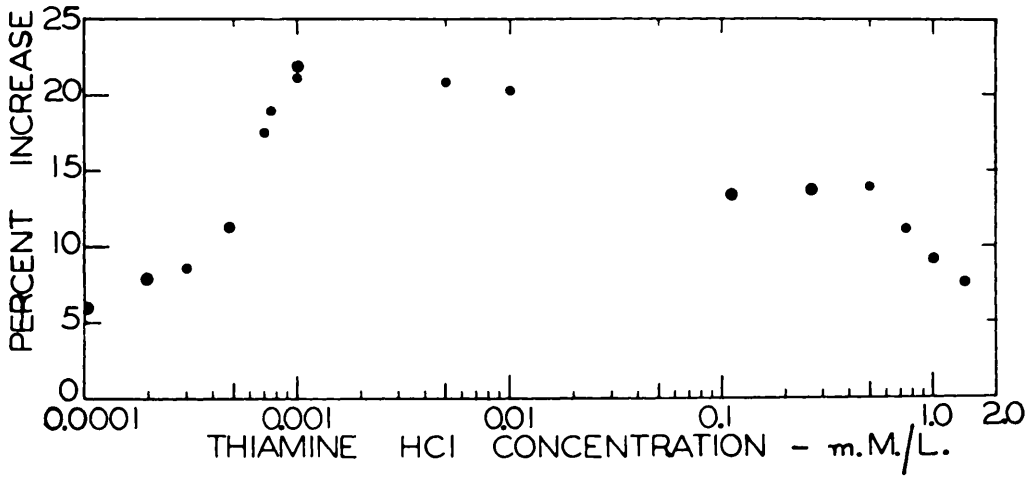

FIG. 2.

Effect of increasing concentrations of thiamine hydrochloride in the perfusion fluid on improvement in work output of frog muscles. Abscissa—thiamine hydrochloride concentration in perfusion fluid, mM per liter. Ordinate—% increase in total work done by muscle perfused with Ringer's solution plus thiamine overwork done by muscle perfused with Ringer's solution alone. Each point is the average of 10 experimental animals. Differences greater than 10% are statistically significant.

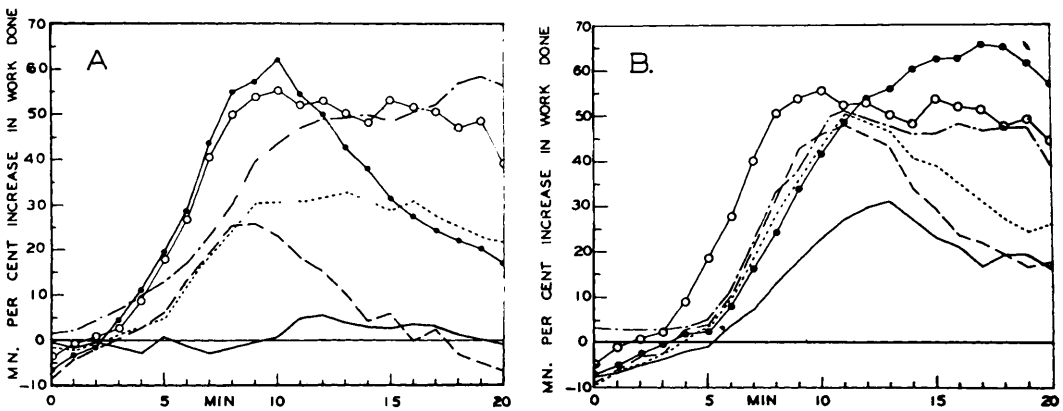

FIG. 3.

Effect of increasing concentrations of thiamine hydrochloride in perfusion fluid on distribution of work outside of frog muscles.

A. ———— Control (no vitamin added to perfusion fluid of second muscle); —— —— —— 0.0002 mM/L thiamine HCl; - - - - - - - - - - - - 0.0005 mM/L thiamine HCl; ——— — ——— 0.00075 mM/L thiamine HCl; ●—●—●—● 0.001 mM/L thiamine HCl; ○—○—○—○ 0.01 mM/L thiamine HCl.

B. Thiamine HCl concentrations of: ○—○—○—○ 0.01 mM/L; ●—●—●—● 0.1 mM/L; ——— — ——— 0.5 mM/L; - - - - - - - - - - - - 0.75 mM/L; —— —— —— 1.0 mM/L; ———— 1.5 mM/L.

Each point is the average of 10 experiments. Differences greater than 15% are statistically significant.

0.001 to 0.1 mM per liter, the maximum improvement in work output is obtained. At higher thiamine concentrations (0.1 to 1.5 mM per liter) the improvement in work output diminishes. Statistical tests showed that in this series of experiments differences in total work output greater than 10% were statistically significant. (See Table I.)

The average percent differences between the height of contraction of the control and vitamin-treated muscles at each minute of exercise are plotted in Fig. 3-A and B. Fig. 3-A shows that as the concentration of thiamine is increased from 0.0002 to 0.01 mM per liter

TABLE II.
Effect of Thiamine Pyrophosphate (Co-carboxylase) on Work Output of Perfused Frog Muscle.

Substance*	Conc. mM/L	Avg work done (N = 10 animals) Cont. leg g cm	Exp. leg g cm	Mean difference† (Exp.-Cont.) g cm	C.R.‡
TP	0.00001	1834	1984	150 ± 73.5	2.0
TP	0.0001	1905	2213	308 ± 60.5	5.1
TP	0.001	1928	2175	247 ± 47.8	5.2
TP	0.01	2072	2348	276 ± 74.3	3.7
TP	0.1	1904	2196	292 ± 75.7	3.9
TP§	0.01	2134	2348	214 ± 49.9	4.3
THCl§	0.01	2107	2286	179 ± 34.2	5.2
THCl + TP‖	0.01	1977	2188	211 ± 45.0	4.7
THCl + TP‖	0.01	1855	2074	219 ± 56.0	3.9
TP + THCl¶	0.01	2212	2321	109 ± 87.5	1.2
TP + THCl¶	0.01	1993	2069	76 ± 52.9	1.4

* TP = Thiamine pyrophosphate. THCl = Thiamine hydrochloride.
† ±1 Standard error of the mean.
‡ Critical ratios greater than 3.0 are statistically significant.
§ Second leg (experimental) perfused with vitamin for only 5 minutes before running fatigue curve instead of 15 minutes as in all other experiments.
‖ Control leg perfused with buffered glucose-Ringer's solution containing 0.01 mM/L thiamine hydrochloride. Experimental leg perfused with the same mixture plus 0.01 mM/L thiamine pyrophosphate.
¶ Control leg perfused with buffered glucose-Ringer's solution containing 0.01 mM/L thiamine pyrophosphate. Experimental leg perfused with the same mixture plus 0.01 mM/L thiamine hydrochloride.

the work output is increased significantly during the later stages of exercise. Fig. 3-B shows that when the thiamine concentration in the perfusing fluid is increased from 0.1 mM per liter to 1.5 mM per liter the improvement in work output diminishes. In Fig. 3-A and B, differences greater than 15% are statistically significant.

Effect of thiamine pyrophosphate (co-carboxylase) on work output. Table II shows the work output when thiamine pyrophosphate was added to the perfusion fluid in concentrations of 0.1 to 0.00001 mM per liter. A significant increase in total work was observed when concentrations greater than 0.00001 mM thiamine pyrophosphate per liter were added to the perfusion fluid. At concentrations greater than 0.001 mM per liter, the increment in work output did not differ significantly from that observed with similar concentrations of thiamine (see Table I). However, the thiamine pyrophosphate was effective at lower concentrations than was thiamine hydrochloride.

Experiments were also performed to see whether the physiological response to thiamine pyrophosphate appeared more rapidly than the response to thiamine alone. For this purpose, the time of perfusion with the vitamin was reduced from 15 to 5 minutes in a series of 10 experiments. The results appear in lines 6 and 7 of Table II, which show that a significant increase in work output can be obtained within 5 minutes with both thiamine pyrophosphate and thiamine hydrochloride. Although the work output was greater with thiamine pyrophosphate than with thiamine hydrochloride, the difference is not statistically significant.

The combined effect of thiamine pyrophosphate and thiamine hydrochloride was tested in the following manner. In one set of 10 experiments, thiamine hydrochloride (0.01 mM/L) was added to the buffered glucose Ringer's solution used to perfuse the first or control leg. Thiamine pyrophosphate was added to this solution for perfusion of the second or experimental leg. In another set of 10 experiments, the order of adding the hydrochloride and the pyrophosphate was re-

versed; *i.e.*, thiamine pyrophosphate was added to the perfusion fluid used for the first or control leg, and thiamine hydrochloride was then added to the perfusion fluid used for the second or experimental leg. The results of these experiments appear in the last four lines of Table II. These experiments, repeated in duplicate, showed that a significant increase in work output was obtained from the addition of thiamine pyrophosphate (0.01 mM/L) to a muscle already treated with an optimum concentration of thiamine hydrochloride. On the contrary, no significant improvement in work output followed the addition of thiamine hydrochloride to a muscle already exposed to an adequate concentration of thiamine pyrophosphate.

Discussion. The results in Table I show that the total work output varies considerably between different groups of frogs. For instance, the average work output of Group a (obtained in March) is considerably less than that of Groups b or c (obtained June and September, respectively). Nevertheless, the differences between the work output of the two legs is of the same order of magnitude. Similarly, the increment in work output obtained after the administration of thiamine is about the same, as shown by duplicate experiments with 0.001 mM thiamine per liter (d and e of Table I; d—runs made in June, e—runs made in November). The large standard errors of the mean differences between work output of the two legs (column 5, Table I) shows that some animals are affected to a much greater degree than others by the administration of the thiamine. The source of this variability in response is not clear from the available data.

The data of Fig. 1 show that the most effective concentration of thiamine for improving work output is 0.001 mM per liter, as compared with 0.01 mM per liter for calcium pantothenate.[2] The maximum improvement in work output is practically the same for each vitamin. Since the normal mammalian blood levels reported in the literature are 0.0003 mM per liter for thiamine[3,4,5,6] as compared with 0.001 mM per liter for pantothenic acid, it is not surprising to find that thiamine improves work output in lower concentrations than does pantothenic acid.[2] The most effective concentration of thiamine was thus 10 to 100 times the normal blood levels.

Evidence that the improvement in work output observed after the administration of thiamine is associated with metabolic processes in the muscle itself is afforded by the observation of improved work output when thiamine pyrophosphate is added to a muscle already receiving an adequate amount of thiamine hydrochloride. The available evidence is inadequate to decide whether this difference is due to differences in permeability of the phosphate and hydrochloride, or to the rate at which the hydrochloride is phosphorylated in the muscle tissue, since it is presumed that thiamine is effective in muscle energy metabolism chiefly as the pyrophosphate—or co-carboxylase.[7]

The present experiments show that maximum improvement in work output occurs with concentrations of thiamine between 0.001 and 0.1 mM per liter. With concentrations greater than 0.1 mM, there is a progressive diminution in effectiveness. A similar phenomenon was observed with calcium pantothenate.[2] Since the pH was maintained at 7.4 even with 1.5 mM per liter thiamine, changes in acidity cannot account for the diminution in physiological effect. Although the data are incomplete, Briem[8] has reported a reduction in contractility of frog muscles at high concentrations of thiamine. Previous investigations have also indicated an increase in the strength of the heart beat at low thiamine concentrations, where high concentrations caused cardiac arrest in the frog.[8,9] Schneider[10] has observed a lethal effect of high vitamin B complex on *Tribolium confusum* Duval in contrast to the

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growth-promoting effect of low concentrations of the same complex. While the mechanism whereby an excess of thiamine or pantothenate may fail to improve work output is problematical, the diminished effect of high concentrations is apparently real.

Summary. The work output of the gastrocnemius muscle of the perfused frog has been determined at different levels of thiamine concentration in the perfusion fluid. The effects on the work output of adding thiamine pyrophosphate (co-carboxylase) and thiamine hydrochloride to the perfusion fluid has been compared. 1. The addition of thiamine hydrochloride to the perfusion fluid improved the work output of frog muscles. The degree of improvement increased with increasing concentrations of hydrochloride from 0.0001 to 0.001 mM per liter. 2. No further improvement in work output resulted when thiamine hydrochloride concentration was increased to 0.1 mM per liter. 3. Increasing the thiamine concentration of the perfusion fluid from 0.1 to 1.5 mM per liter progressively reduced the improvement in work output. 4. Thiamine pyrophosphate was also effective in increasing the work output in perfused frog muscles. 5. Thiamine pyrophosphate increased the work output at lower concentrations than did thiamine hydrochloride. 6. At concentrations of 0.001 mM per liter or above, the extent of the improvement in work output was no greater with thiamine pyrophosphate than with thiamine hydrochloride.

15037

X. Carcinoma of Mammary Gland Following Injection of Methylcholanthrene into Mice of NHO Strain.

LEONELL C. STRONG.*

From the Department of Anatomy, Yale University.

Adenocarcinomas of the mammary gland in mice have been known since 1854.[1] The usual degree of morphologic variation exhibited by mouse mammary gland tumors has been interpreted by Apolant,[2] and others,[3] as representing a series of sequential phases of a single pathological process. A continuous gradient may be observed from the benign adenomas to the adenocarcinomas and the undifferentiated medullary carcinomas. In general these tumors are only histologically malignant, with remote metastases and extensive invasion of adjacent tissues occurring only infrequently.

Numerous agents have been incriminated by the many investigators of the problem as having some influence on the origin of these tumors in mice. However, 5 influences have received rather widespread recognition. These influences may be briefly classified as (1) genetic; (2) hormonal; (3) the "milk" influence; (4) dietary factors; and (5) somatic mutation.

Recently mammary gland tumors which had been encountered only in genetically susceptible mice, either spontaneously or following hormone injection, have been duplicated following the subcutaneous injection of methylcholanthrene in sesame oil into female mice of certain strains which lack the milk influence, are spontaneously resistant to mammary gland tumors, and do not develop such tumors after estrogen treatment.[4,5,6] This observa-

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